Serum Total Sialic Acid as a Novel Complementary Candidate Marker of Hepatic Damage in Obstructive Jaundice

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Abstract. Background: The present study was carried out to investigate oxidant-antioxidant status and serum total sialic acid (SA) levels as alternative markers complementary to routine laboratory tests in an experimental obstructive jaundice model (OJM). Rats were divided into three groups: sham operated control (SOC), OJM monitored for 7 days (OJM-7), and OJM monitored for 14 days (OJM-14). Antioxidant activities, lipid peroxidation, C reactive protein (CRP), and SA concentrations were analyzed at the end of post-operative days 7 and 14. In both OJM groups, SA and CRP levels were significantly increased when compared with the SOC group. Moreover SA and CRP levels were significantly correlated in both groups. As a marker of inflammation and oxidative stress in obstructive jaundice, serum SA may serve as an adjunct when combined with other markers in disease screening and progression.

Key words: Sialic acid, C reactive protein, cholestasis, oxidative stress, antioxidants, rat.

Introduction

Obstructive jaundice (OJ), caused by the structural and functional impairment of the hepatobiliary system, is seen in many clinical situations, including benign tumor or stricture of the bile duct, gallstone complications of pancreatitis, or biliary surgery. OJ may cause serious complications like sepsis, immune depression, coagulopathy, wound breakdown, gastrointestinal hemorrhage, and cardiovascular, hepatic, and renal failure [1]. Several mechanisms have been suggested for the cytotoxicity of bile acids accumulated in hepatocytes due to the obstruction of the biliary tract, among them detergent properties, alteration of intracellular Ca²⁺ homeostasis, and impairment of mitochondrial respiration [2]. Accordingly, increasing concentrations of the bile acids were shown to decrease mitochondrial membrane potential developed upon succinate energization, decrease state 3 respiration and enhance state 4, leading to enhanced permeability of mitochondria to protons [2]. Moreover, together with inflammatory processes, free radicals, oxidative stress and lipid peroxidation are associated with liver damage produced by bile flow obstruction [3].

The sialic acids (SA) are a family of 9-carbon carboxylated sugars usually found as terminal monosaccharides of animal oligosaccharides and are commonly positioned at non-reducing terminal of complex carbohydrates[4,5]. Although the mechanisms underlying the elevated SA concentrations in different diseases are not clear, SA has been used as laboratory marker in a variety of pathological conditions and proposed as a valuable indicator for inflammatory diseases [6-8]. Accordingly, the acute phase protein C- reactive protein (CRP) was also determined as a sensitive indicator useful for diagnostic and prognostic assessments of inflammation [9].

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In this paper we intended to examine oxidant-antioxidant status, CRP, and serum SA concentrations as alternative markers complementary to routine laboratory tests in a cholestatic rat model.

**Materials and Methods**

**Animals and experimental design.** All animal protocols were approved by the committee on the use of live animals in teaching and research of Istanbul University. A total of 27 female Sprague Dawley rats aged 8 weeks at the beginning of the experiment (body weight, 200 – 250 g) were used. The animals were obtained from the Istanbul University Experimental Medicine Research Institute Animal Laboratory. Animals were housed in cages in a room with a controlled environment (room temperature, 22±2°C; relative humidity, light/dark cycle, 12h/12h) with free access to food and water.

Rats were divided into three groups. The first group was sham operated control (SOC), the second underwent bile duct ligation (BDL) and was monitored for 7 days, and the third underwent BDL and was monitored for 14 days. Indices of hepatic damage were evaluated 7 days and 14 days after surgery.

For both OJM groups, under ketamine anaesthesia (40 mg / kg, intraperitoneal), the common bile duct was isolated, doubly ligated in its middle third with a 4 - 0 silk suture, and transected between the two ligatures. The abdominal cavity was closed with an operation in which only the bile duct dissection, not the ligation, was performed. In all groups the incision was closed with a 4 - 0 silk running suture according to the method previously described by Yorgancı et al. [10]. OJM-7 group was sacrificed 7 days after surgery and the OJM-14 group was sacrificed 14 days after surgery. The rats were sacrificed by cervical dislocation. Blood samples were taken by intracardial puncture after thoracotomy.

**Blood samples.** Blood samples in plain tubes were centrifuged at 3000 rpm for 10 min to extract serum and in heparinized tubes at 2000 g for 10 min to obtain plasma, which was stored at -20°C for measurements. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin levels were measured with an autoanalyzer (Abbott C8000, Abbott, U.S.A) to evaluate the liver injury and the state of cholestasis.

**Determination of Sialic Acid.** SA levels were measured in serum using Warren’s thiobarbituric acid assay [11]. Samples were incubated with 0.1 N H₂SO₄ at 80°C for 1 hour. SA levels were determined in hydrolysate. In this colorimetric assay, sialic acids are oxidized with sodium periodate in concentrated phosphoric acid. The periodate oxidation product is coupled with thiobarbituric acid and the resulting chromophore is extracted into cyclohexanone. Results were expressed in mg/dL.

**Determination of CRP.** Serum high-sensitivity C-reactive protein (hs-CRP) levels were measured using a nephelometric method (Catalog no: 0QIY BN II Nephelometer Dade Behring, Marburg, Germany). Results were expressed in mg/L.

**MPO activity.** The determination of serum myeloperoxidase (MPO) activity depends on the reduction of o-dianisidine. Reduced o-dianisidine was measured at 410 nm by spectrophotometer [12]. Results were expressed in U/mL.

**Lipid Peroxidation assay.** An end product of lipid peroxidation, malondialdehyde (MDA) content in serum was determined according to the method of Yagi [13]. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56 x 10⁵ M⁻¹ cm⁻¹. Results were expressed as nmol MDA/mL.

**Total Antioxidant Activity.** Total antioxidant activity (TAA) was determined in plasma by the method of Koracavic et al. [14]. A standardized solution of Fe-EDTA complex reacts with H₂O₂ by a phenton-type reaction leading to the formation of hydroxyl radicals (OH'). These ROS degrade benzoate, resulting in the release of TBARS. Antioxidants from the plasma samples cause suppression of the production of TBARS. Finally, absorbance values were measured by a spectrophotometer at 532 nm. Results were expressed in mmol /L.

**SOD Activity.** Superoxide dismutase (SOD) activities were assayed by its ability to increase the effect of riboflavin-sensitized photooxidation of orthodianisidine [15]. The activity of superoxide is generated by illuminating the reaction mixture containing orthodianisidine and riboflavin by light from a fluorescent lamp. The oxidation of orthodianisidine, as sensitized by riboflavin, is enhanced by SOD. The increase is linearly dependent on SOD concentration. The absorbance of the colored product is evaluated by spectrophotometry at 460 nm. Results were expressed in U/mL.
Determination of GSH. Serum reduced glutathione (GSH) concentration was determined according to the method of Beutler et al. [16], using metaphosphoric acid for protein precipitation and 5-5-dithiobis-2-nitrobenzoic acid for color development. GSH levels were calculated using an extinction coefficient of 1.36x10^4 M$^{-1}$ cm$^{-1}$. Results are expressed in mg/dL.

Catalase Activity. The catalase activity was measured spectrophotometrically at 240 nm according to the method of Aebi [17]. This method was based on the hydrolyzation of H$_2$O$_2$ and decreasing absorbance at 240 nm. The conversion of H$_2$O$_2$ into H$_2$O and 1/2 O$_2$ in 1 min under standard conditions was considered to be the enzyme reaction velocity. Results were expressed in U/mL.

Statistical Analysis. The differences between tested and control groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons. Values with p<0.05 were considered statistically significant. Pearson coefficient correlations were used to perform the correlation analysis.

Results

OJ was determined by blood assays on postoperative days 7 and 14, and was confirmed in all animals in OJM-7 and OJM-14 groups. BDL caused significant increases in serum bilirubin, AST, and ALT. No significant difference was observed in serum bilirubin, AST, and ALT levels in OJM-7 and OJM-14 groups. The results of the liver function tests are presented in Table 1.

Significant increases were observed in SA and CRP levels in the OJM-7 and OJM-14 groups when compared with the SOC group (p<0.001, p<0.05 and p<0.01, p<0.001 respectively). SA levels were significantly correlated with CRP levels in the OJM-7, OJM-14, and the SOC groups (r=0.889, p<0.01; r=0.816, p<0.01; r=0.883, p<0.01) (Table 2).

BDL produced significant increases in the serum MPO activity and MDA levels in the OJM-14 group compared with the SOC group (P<0.05) (Table 2).

In the OJM-14 group, BDL led to significant reductions in TAA and rGSH levels when compared with the SOC group (p<0.01). On the other hand, SOD levels were significantly decreased in both the OJM-7 and OJM-14 groups compared with the SOC group (P<0.05, P<0.001) (Table 2).

When CAT levels were compared in the three groups, a significant decrease was observed in the OJM-14 group compared with the OJM-7 group (P<0.01) (Table 2).

Discussion

Despite the advances in laboratory techniques, the need for suitable adjunct biochemical markers for the detection of hepatic damage persists. Therefore, the identification of novel complementary biochemical markers for OJ remains an important goal.
for many laboratories around the world. To our knowledge, the oxidant-antioxidant status has not been evaluated with inflammatory markers in an OJM model before. In agreement with the literature, rats in our study, subjected to BDL for 7 and 14 days, exhibited increased bilirubin, AST, and ALT levels, indicating the presence of cholestasis and hepatic injury [2,18]. Serum SA levels were found to be significantly higher in both OJM-7 and OJM-14 groups when compared with the SOC group. Moreover, SA and CRP levels were significantly correlated in both groups. In the OJM-14 group, BDL induced an oxidative stress as evidenced by significantly increased MDA, decreased TAA, and reduced GSH and antioxidant enzymes including SOD and CAT. On the other hand, in the OJM-7 group, only the decrease in SOD activity was statistically significant when compared with the SOC group.

Cholestasis, or impaired bile flow, is a common pathophysiological process in many human diseases that causes hepatic damage by retention and accumulation of toxic hydrophobic bile salts, inducing persistent inflammation and oxidative stress [1,18]. Enhanced production of reactive oxygen intermediates species lipid peroxidation by disturbing oxidant-antioxidant balance in hepatic mitochondrial fraction [7,19]. MDA, the most important indicator of lipid peroxidation, was found to be significantly increased in the OJM-14 group compared with the SOC group. On the other hand, the increase in the MDA levels of the OJM-7 group was statistically insignificant. Consistent with our findings, Parola et al. [20] reported that lipid peroxidation was a relatively late event, starting after 1-2 weeks of BDL.

Studies in humans and animals with biliary obstruction suggest that inflammation plays a central role in hepatic damage [6,10]. SA, abundantly present in all biological membranes, has been reported to increase in human and animals during a number of pathological situations in which the contributory event is either tissue damage, tissue proliferation, or inflammation, probably resulting from increased levels of richly sialylated acute-phase glycoproteins [6,21]. Multiple factors affecting the sialylation and desialylation of glycoproteins and glycolipids may increase or decrease the amount of SA in serum.

**Table 2. The results of the oxidative stress, antioxidants and inflammatory biomarkers in blood.**

<table>
<thead>
<tr>
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<th>BDL</th>
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<tbody>
<tr>
<td></td>
<td>SOC group (n =9)</td>
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<tr>
<td>SA (mg/dL)</td>
<td>42.29 ± 15.21</td>
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<tr>
<td>CRP (mg/L)</td>
<td>2.49 ± 1.68</td>
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<tr>
<td>MPO (U/mL)</td>
<td>0.09 ± 0.04</td>
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<tr>
<td>MDA (nmol MDA/mL)</td>
<td>1.11 ± 0.32</td>
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<tr>
<td>TAA (mmol/L)</td>
<td>1.62 ± 0.15</td>
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<tr>
<td>rGSH (mg/dL)</td>
<td>1.24 ± 0.25</td>
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<tr>
<td>SOD (U/mL)</td>
<td>48.05 ± 2.78</td>
</tr>
<tr>
<td>CAT (U/mL)</td>
<td>1458 ± 499.8</td>
</tr>
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</table>

Kruskal - Wallis followed by Dunn’s multiple comparisons were used to determine the differences between the groups. Data are expressed as mean ± SD; <sup>a</sup>P<0.05 significantly different from SOC group; <sup>b</sup>P<0.01 significantly different from SOC group; <sup>c</sup>P<0.01 significantly different from OJM-7 group. BDL: Bile duct ligated groups, SOC: sham-operated control group, OJM-7: Obstructive jaundice model group monitored for 7 days; OJM-14: Obstructive jaundice model group monitored for 14 days; SA: Sialic acid, CRP: C - reactive protein, MPO: myeloperoxidase, MDA: malondialdehyde, TAA: total antioxidant activity, rGSH: reduced glutathione, SOD: superoxide dismutase, CAT: catalase.
urine, and other body fluids [22]. Consequently, with respect to our results the rise in serum SA may be attributed to the liberation of SA residues from damaged hepatocyte cell membranes into circulation.

It has been shown that endotoxemia in OJ activates immunocompetent cells, such as monocytes, macrophages, and endothelial cells, to produce a variety of cytokines that contribute to an uncontrollable inflammatory cascade, causing multiple organ dysfunction or even death [23]. Serum CRP, an acute-phase reactant, is considered as a sensitive indicator useful for diagnostic and prognostic assessments of inflammation [5,18]. Consistent with the literature [23], serum CRP levels were increased in both OJM groups.

In the present study, serum SA levels were significantly correlated with CRP levels in both experimental OJM groups. Based on our data, we suggest that in clinical medicine SA concentrations seem to be dependent on liver status in OJ and it might therefore serve as an adjunct when combined with other markers in disease screening, disease progression follow-up, and in the monitoring of treatment response. Baba et al. [24] investigated the potential of SA in inflammatory states and reported elevated serum total SA in patients suffering from Crohn’s disease to be somewhat more sensitive markers of ulceration than CRP. In contrast, Kosokai [25] concluded that SA appeared to be sufficiently useful as a parameter of inflammation independent of CRP, demonstrating increased SA concentrations in patients with rheumatoid arthritis. However, in the literature it is also suggested that SA is a non-specific marker for a given disease, and that some non-pathological factors, such as aging, pregnancy and smoking, may have an effect on SA concentrations. This may limit the potential clinical usability of SA determination [6].

Prominent among the inflammatory cells that invade obstructed livers are neutrophils, which have been shown to infiltrate the liver within three hours of BDL and remain there for days to weeks as fibrosis progresses [26]. MPO, a peroxidase enzyme found primarily in neutrophil granules, is released as a response to various stimulatory substances. Measuring the activity of this neutrophil-specific enzyme has been used to define the role of neutrophils in tissue injury [27]. Laschke et al. [28] suggested that BDL produced a significant increase in MPO activity that indicates the leukocyte accumulation in the rat liver tissue compared to the controls. Likewise, compared with the SOC group, we observed a significant increase in the MPO activity of the OJM-14 group. In contrast, the increase in the MPO activity of the OJM-7 group was not statistically significant. In accordance with previous reports, the data of this study show an association between experimental OJ and oxidative stress in the plasma of OJM rats [18-20]. It has been previously shown that hepatic mitochondria generate ROS when isolated hepatocytes are exposed to hydrophobic bile acids; therefore, free radical production from mitochondria during cholestasis is likely the mechanism of cholestatic liver injury [3]. In our study, increased MPO activities were evidenced by the significant increase in plasma MDA content and by the decrease in TAA. Increased serum MPO due to chronic biliary obstruction indicates that cholestatic injury involves the contribution of neutrophils, and we may propose that neutrophil-derived oxidative tissue damage is evident in our experimental model.

When SOD, CAT activities and TAA and rGSH levels were evaluated in the OJ-induced rats, SOD was found to be the only antioxidant enzyme which decreased significantly both in OJM-7 and OJM-14 groups. Cu/Zn SOD, the key enzyme in the metabolism of oxygen free radicals, is capable of eliminating free radicals produced by inflammatory cytokines and preventing damage from peroxides [29]. Gong et al [30] reported that infection with a retrovirus carrying SOD genes significantly reduced hepatocyte injury and concluded that gene delivery of SOD is a promising method of reducing the injury caused by cholestatic diseases because of its unique subcellular location. Karaman et al. [18] reported that ligation of the bile duct produced a significant reduction in SOD and CAT activities in liver after 7 days when compared with control and sham groups. Roeb et al. [31] showed depleted GSH levels in the liver samples of BDL rats and
suggested that GSH is involved in the acute phase reaction during obstructive cholestasis. Similarly, in the present study, the decreases in serum CAT activities and TAA and rGSH levels were significant on day 14. The disturbance of the oxidant–antioxidant balance has been suggested to be responsible for cholestatic liver injury and on the other hand CAT and SOD enzymes act as hepatoprotective, playing important roles in preventing oxidative stress [18,19].

In conclusion, reduced antioxidant activity in the OJM rats might increase the susceptibility to liver injury by oxygen-derived free radicals. SA might serve as an adjunct when combined with other markers in disease screening and in monitoring the treatment response in OJ.

References